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Myeloperoxidase in the Progression of Breast Cancer

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14. ABSTRACT

A high myeloperoxidase (MPO) expression genotype is strongly associated with better survival among women with breast cancer that undergo chemotherapy. The objective of this project is to find a mechanistic basis for this clinical observation. We examined the effect of human-type MPO expression in a mouse model for human breast cancer. We have crossed mice that carry the polyoma virus middle T oncogene (PyMT) and develop mammary tumors with mice that carry a human MPO (huMPO) transgene. We observed no difference in time of tumor onset or multifocal disease between PyMT/wt and PyMT/huMPO mice. However, huMPO results in reduced tumor growth. Most but not all mice of either genotype developed macroscopic lung metastasis. PyMT/huMPO mice have fewer metastatic foci than PyMT/wt mice and the difference is consistent with their smaller tumor burden. We found huMPO expressed in tumor cells in mammary tumors and metastases in PyMT/huMPO mice. We have considered different mechanisms by which MPO may be protective in breast cancer. One hypothesis was that MPO expressed in tumor-associated macrophages contributes to macrophage-mediated tumor suppression and augments the effect of chemotherapy. We found no evidence for a role of cytotoxic macrophages in the tumor suppressive effect of MPO. In contrast our data show that cytotoxic drugs and possibly other pro-apoptotic factors induce MPO expression and that MPO expressed in tumor cells appears to contribute to cell death in the presence of cytotoxic drugs. These results can provide the rationale to search for non-toxic treatments that induce MPO and that could be used in synergy with low dose chemotherapy protocols.

15. SUBJECT TERMS
Breast Cancer, Animal Model, Myeloperoxidase, Tumor Progression, Chemotherapy

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Table of Contents

INTRODUCTION:

Myeloperoxidase (MPO) is an oxidant-generating enzyme that is abundant in leukocytes. It reacts with hydrogen peroxide and chloride to produce hypochlorous acid (HOCl). The MPO/HOCl pathway also generates secondary reactive oxygen and nitrogen species. In mice expression of MPO is restricted to neutrophils whereas human MPO (huMPO) is more broadly expressed including in monocytes and macrophages. In humans a functional -463GA promoter polymorphism determines expression levels with the G-allele resulting in higher expression than the A-allele. Through the production of toxic molecules MPO plays an important protective role in the defense against invading microbes. In mouse models expression of human MPO also contributes to inflammatory diseases such as atherosclerosis and Alzheimer's disease. In humans high MPO expression – determined by analyzing the -463GA promoter polymorphism – has been linked to an increased cancer risk particularly for lung cancer in smokers. Analyzing the outcomes of two independent clinical trials in breast cancer Ambrosone and colleagues found that the high MPO expression genotype is strongly associated with better survival among women treated with chemotherapy and tamoxifen (1, 2). If MPO is protective in women with breast cancer, modulation of MPO expression and activity may be desirable. The objective of this project is to directly test the role of MPO in breast cancer progression using transgenic huMPO expressing mice and a transgenic mouse model for mammary tumorigenesis. This project will also examine mechanisms by which MPO may be beneficial.

BODY: -- *All figures are in the Appendix*. --

Task #1: Generate the mouse strains required for this project by crossbreeding

We have breed female mice with one copy of G-allele of the human MPO (huMPO) transgene with male mice with the PyMT transgene. We have generated sufficient numbers of female PyMT/wt and PyMT/huMPO-G mice for experiments to study the role of MPO in mammary tumor development and progression.

To obtain cell lines to study both the human G and A-allele in PyMT mammary tumor cell lines we have also breed mice with the A-allele of the human MPO transgene with PyMT mice. We have generated littermate females that are either PyMT/wt or PyMT/huMPO-A and have established cell lines from mammary tumors of these mice.

Breeding of huMPO-G/moMPO$^{-/-}$ and moMPO$^{-/-}$ mice with immune deficient Rag2 to obtain Rag2/huMPO-G/moMPO$^{-/-}$ and Rag2/moMPO$^{-/-}$ mice has proven difficult. We have obtained much fewer than the expected number of litters and the number of pubs per litter is very small (2-4). It is unclear at this time whether the breeders have reduced fertility or whether the majority of embryos is lost in utero. We have not been able to generate sufficient numbers of MPO deficient mice in an immune-deficient background to address the role of mammary tumor cell MPO in a xenograft model.

Task #2: Determine the incidence, growth, progression and metastasis of PyMT mouse mammary tumors in mice expressing the human MPO gene or lacking MPO expression

Tumor development: Mammary tumor development in cohorts of female PyMT/wt and PyMT/huMPO-G mice is summarized in Table 1. All animals in the experimental cohorts develop multifocal mammary tumors. We observed no difference between the groups in the time of appearance of palpable tumors or in the number of mammary glands involved. Mammary tumor growth is somewhat attenuated in PyMT/huMPO-G mice as illustrated by the difference in cumulative tumor burden per mouse shown in Figure 1, however the difference in cumulative tumor burden between PyMT/wt and PyMT/huMPO-G mice was

Table 1 Mammary Tumor Development in PyMT Mice expressing huMPO

	PyMT/wt	PyMT/huMPO-G	*P*
Tumor Development	20/20 mice	20/20 mice	
Mammary Tumor Onset (weeks of age)	12.6 ± 1.9	13.1 ± 2.6	
Tumor Incidence (# glands affected)	4 - 8	4 - 8	
Cumulative Tumor Burden (mm^3 at 23 woa)	6285 ± 1486	3971 ± 1310	0.06

Male PyMT mice were crossed with females carrying one copy of the human MPO-G transgene. Tumor development was followed in cohorts of virgin, female littermates. All mice were in the C57Bl/6 background.
P - Probability of no difference using two-tailed t-test

not statistically significant. These data indicate that huMPO does not affect tumorigenesis in the PyMT model and has a modest effect on tumor growth.

Metastasis: In the PyMT model metastasis from the mammary tumors to the lungs is observed frequently and the number of metastatic tumors is a function of the size of the primary mammary tumors (3). To evaluate metastasis in PyMT/wt and PyMT/huMPO-G mice lungs from tumor-bearing female mice at 23 weeks of age are harvested and processed for analysis. The left lobes of the lungs of each mouse were fixed and metastatic tumors on the lung surface are counted under a low magnification microscope. The right lobes of the lungs were flash-frozen and metastatic tumor burden was determined by amplifying the PyMT transgene in RNA from lung tissue as described (3). PyMT message from cultured PyMT cells was processed in parallel and the number of PyMT positive cells in the lungs was calculated based on the results from the PyMT cell control. Lung metastasis was observed in 85% of PyMT/wt mice and 75% PyMT/huMPO-G mice (table 2). The number of metastatic foci and

Table 2 Lung metastasis in PyMT/wt and PyMT/huMPO-G mice

	PyMT/wt	PyMT/huMPO-G	*P*
Macroscopic metastases	17/20 mice	15/20 mice	
# macroscopic foci/mouse Median (± Range)	6 (0-104)	4 (0-59)	0.024
Lung PyMT RNA	20/20 mice	20/20 mice	
# tumors cells/mouse Median (± Range)	1.9 x10E3 (2x10E2 - 8x10E5)	1.1x10E3 (2x10E2 - 3.5x10E5)	0.045

Mice were killed at 23 wk of age. Number of macroscopic foci was counted on right lungs; PyMT RNA was quantified by q-PCR and number of tumors cells was caluculated based on standard curved derived from cultured cells.
P - Probability of no difference using non-parametric Mann-Whitney U test

the number of PyMT-positive cells in PyMT/huMPO mice were smaller than in PyMT/wt mice. The difference in lung metastasis is consistent with the somewhat smaller overall tumor burden in PyMT/huMPO-G mice. It may also suggest that MPO-expression is detrimental to the survival of metastatic cells in the early stages of metastasis. This will be addressed in future studies by comparing lung metastasis after intravenous injection of different doses of MPO-positive and MPO-lacking breast cancer cells.

Human MPO expression: HuMPO is expressed in a subset of tumor cells in mammary tumors from PyMT/huMPO-G mice. Immunohistochemistry often detects huMPO at the periphery of the tumor HuMPO is expressed in a mosaic fashion (figure 2). Interestingly, MPO is primarily expressed in tumor cells, as demonstrated by histology and by costaining with PyMT antigen (Figure 3). Immunohistochemistry also detects huMPO expression in some, but not all, lung metastases in PyMT/huMPO-G mice. Similar to what we observed in mammary tumors, lung metastases express huMPO in a mosaic pattern in tumor cells (Figure 4). High huMPO expression in a subset of cells suggests a threshold effect in which a specific signal induces huMPO. In this regard our data show that huMPO staining coregisters with the apoptosis marker, cleaved caspase-3 (Figure 5). Together these data demonstrate that in PyMT/huMPO-G mice huMPO is expressed in a subset of tumor cells both, in mammary tumors and in metastasis, which is reminiscent of MPO expression in human tumor tissues. It also demonstrates a connection between MPO expression and apoptosis and suggests that the subset of tumor cells expressing MPO is more susceptible to cell death or that initiation of apoptosis leads to MPO expression with MPO potentially serving as an effector molecule in the apoptotic process.

Cell lines: For further study of human MPO in tumor cells we have established a number of cell lines with and without huMPO expression from PyMT mammary tumors. These cell lines are listed and described in our 2011 progress report.

Task #3: Determine the growth and metastasis of transplanted human breast tumors in immune deficient mice that express the human MPO gene

These experiments have not been initiated due to difficulties breeding $MPO^{-/-}$ mice into the immune deficient RAG-2 strain. See above.

Original Task #4: Determine whether MPO produced by macrophages kills breast cancer cells

Our data presented and discussed in the 2011 progress report did not support the hypothesis that huMPO produced by cytotoxic M1-type macrophages generates toxic molecules that damage breast cancer cells and works in synergy with other cytotoxic or cytostatic agents.

Revised Task #4: Determine whether MPO contributes to tumor cell death induced by chemotherapy drugs

We tested the hypothesis that chemotherapy drugs induce MPO expression in breast cancer cells and that in turn production of reactive oxygen species by MPO augments chemotherapy induced cell death. This mechanism would be consistent with the observation that chemotherapy is highly effective in women with the high MPO G-allele.

Effect of chemotherapy drugs on MPO expression in vitro: We determined huMPO expression in human and PyMT breast cancer cell lines in response to 5-fluoro-uracil (5-FU), doxorubicin (dox) and cyclophosphamide (CP). The following cell lines were tested: the PyMT/huMPO cell lines J219G, J357G andJ395A, the PyMT/wt cell lines J289 and J381, the human tumor cell lines MCF-7 and MDA-MB-231 and the human mammary epithelial

cell line MCF-10A. Cells under cell culture conditions were treated for 72 hours with vehicle, 50FU, Dox or CP. Then cells were suspended, permeabilized and stained with anti-human MPO-FITC or a control antibody-FITC and analyzed by flow cytometry. Figure 7 in our 2011 progress report shows representative histograms of FACS analysis. Flow cytometry data are summarized in table 3. We found that J289 and J381 cells, as expected had no detectable huMPO under any conditions. Untreated J219G, J357G andJ395A cells expressed considerable levels of huMPO and huMPO expression increased in a dose-dependent fashion in the presence of 5-FU or Dox. HuMPO did not increase in the presence of CP (not shown) and CP was not cytotoxic for our panel of cell lines at any concentration tested. Untreated human breast cell lines had no detectable MPO and both, 5-FU and Dox increased huMPO expression. Untreated human cell lines had no detectable huMPO. These data demonstrate that MPO expression is upregulated in breast cancer cells exposed to cytotoxic drugs.

Table 3 Cytotoxic Drugs Upregulate MPO Expression in Breast Cancer Cell Lines

	Untreated		**5-FU (1 uM)**			**5-FU (10 uM)**			**Dox (5 uM)**			**Dox (20 uM)**		
	MPO expression		MPO expression			MPO expression			MPO expression			MPO expression		
Cell line	mean	SD	mean	SD	P	mean	SD	P	mean	SD	P	mean	SD	P
J219G	48.7	9.4	68.9	10.2	ns	123.5	14.8	0.012	77.1	20.1	0.05	145.6	15.6	0.001
J357G	63.5	12.3	101.3	13.7	ns	189.5	19.9	0.01	117.9	12	0.045	188.2	17.1	0.001
J395A	29.9	11	57.9	12.2	0.047	225.8	31.9	0.001	64.9	12.8	0.04	223.1	30.7	0.001
J289	9.5	3.1	9.3	3.1	ns	9.2	3.4	ns	9.4	3.2	ns	9.7	3.1	ns
J381	8.7	3.2	8.7	3.1	ns	8.9	3.3	ns	8.8	2.9	ns	8.5	3.2	ns
MCF7	6.5	2.6	11.5	4.4	0.035	99.5	4.6	0.001	12.6	2.4	0.05	87	7.5	0.002
MCF10A	8.3	3.4	24.9	8.5	0.001	28.7	10.3	0.001	23.8	5.7	0.001	26.9	9.9	0.001
MDA-MB-231	7.1	2.3	14.7	4.5	0.05	42.5	9.9	0.001	13.3	4.5	ns	23.7	9.8	0.01

P - probability of no difference from untreated control

Does MPO activity contribute to cell death in breast cancer cells? We had shown that that MPO-expressing mammary tumor cells, J357G and J395A, are more susceptible to 5-FU in vitro than cells unable to express MPO, J211 and J289 (figure 6). Similarly a recent publication demonstrated that MPO expression determined cytotoxicity of the anti-leukemic drug parthenolide in AML cells (reference 6). If MPO contributes as an effector to cell death, one would expect that inhibition of MPO activity would reduce the effect of cytotoxic drugs. 4-aminobenzoic acid hydrazide (4-ABH) is a small molecule peroxidase inhibitor that has good specificity for MPO. We tested 4-ABH on 5-FU and Dox cytotoxicity. The data were difficult to interpret as 4-ABH by itself at various concentrations was cytotoxic for the breast cancer cell lines. We are now testing the role of MPO in apoptosis by using MPO-specific small interfering RNA (siRNA). We are in the process to optimize conditions for siRNA inhibition of MPO expression and cell death experiments have not been completed at this time.

Effect of chemotherapy drugs on MPO expression and tumor growth in vivo: We have set up a model in which we subcutaneously implant small pieces of mammary tumors from PyMT/wt or PyMT/huMPO-g mice into C57Bl6 mice. These tumors grow progressively and their histology resembles that of the mammary tumors in the transgenic mice. In this model, we treated tumor-bearing mice with a single dose of 5-FU (50mg/kg) and tested the tumors for MPO expression 5 days later. As shown in figure 7, MPO is detectable by immunostaining in untreated PyMT/huMPO-G tumors (panel A) and expression increases

markedly after treatment with 5-FU (panel B and C). In a proof of principle experiment we also determined the response of PyMT/wt and PyMT/huMPO-G tumors in mice to systemic chemotherapy with 5-FU. Mice were implanted with tumors and 5FU (50mg/kg) was injected subcutaneously once a week for 3 weeks. 5-FU treatment reduced tumor size for both, PyMT/wt and PyMT/huMPO-G tumors (figure 8). However the effect on PyMT/huMPO-G tumors was more pronounced and longer lasting. These data suggest that MPO activity contributes to cell death induced by 5-FU therapy.

KEY RESEARCH ACCOMPLISHMENTS:

- A human MPO transgene reduces mammary tumor growth in PyMT mice.
- In the PyMT model human MPO is expressed in mammary tumors, in cell lines established from these tumors and in metastasis.
- The MPO product HOCl is toxic for breast cancer cells albeit at very high concentrations when added exogenously.
- Macrophages can kill breast cancer cells but we found no evidence for a role of MPO.
- 5-FU and doxorubicin induce human MPO expression in breast cancer cell lines.
- 5-FU induces human MPO expression in breast cancer tissue in vivo.
- MPO expression correlates with increased sensitivity to chemotherapy.

REPORTABLE OUTCOMES:

- Mueller BM, Maki R, Reynolds WF Myeloperoxidase in the progression of breast cancer. Abstract BC083927-3263, Era of Hope, August 2-5, 2011, Orlando FL
- We have established a number of mammary tumor cell lines from human MPO transgenic and control PYMT mice. We are currently characterizing these cell lines and will make them available to interested investigators.
- A manuscript reporting our findings on MPO in breast cancer cells is in preparation.
- We are planning to submit an R01 further exploring the role of MPO in drug-induced apoptosis and in the augmentation of cytotoxic therapies.

CONCLUSION:

Whereas tumor onset is not delayed in PyMT mice expressing huMPO compared to PyMT/wt mice, tumor growth is attenuated. Metastasis is similarly inhibited to some extent. The marginal effect of the presence of human MPO is not unlike the situation in women with breast cancer where the high expressing genotype confers a strong survival advantage in women who received adjuvant chemotherapy but not in those that did not receive chemotherapy.

We have considered a number of different mechanisms by which MPO may be protective in breast cancer. One hypothesis was that MPO expressed in tumor associated macrophages would contribute macrophage-mediated tumor suppression and augment the effect of cytotoxic therapy. We have not found evidence for cytotoxic macrophages contributing to a tumor suppressing effect of MPO. In contrast we have found that MPO expressed in the tumor cells themselves appears to contribute to cell death in the presence of cytotoxic drugs. This potentially important observation can provide the rationale to search for non-toxic treatments that induce MPO and that could be used in synergy with low dose chemotherapy protocols.

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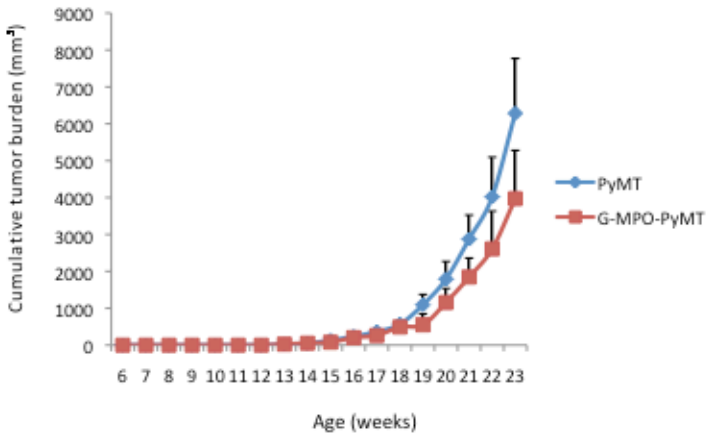
APPENDIX:

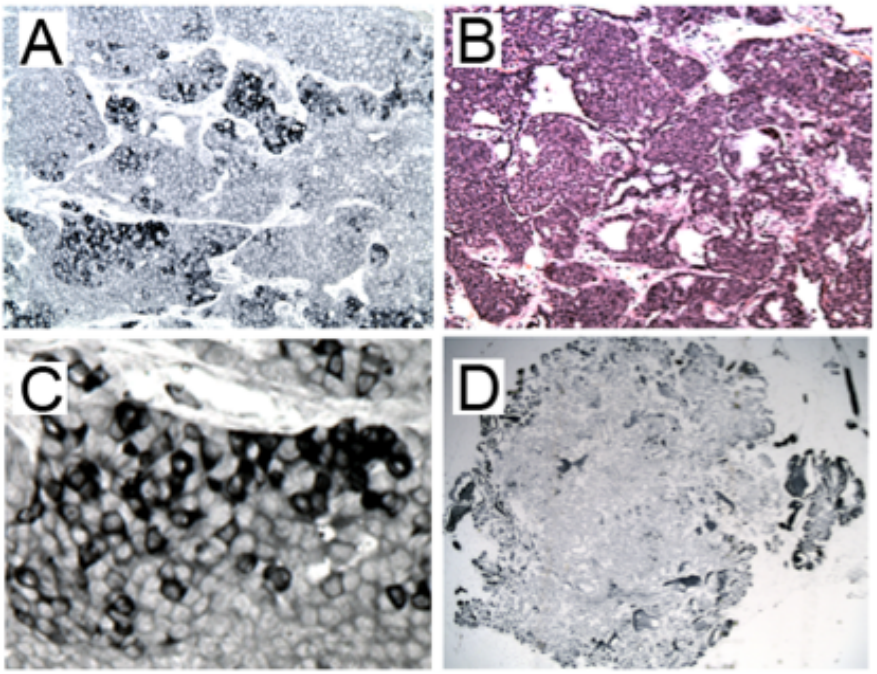
Figure 1-7

Figure 1 Mammary Tumor Burden in PyMT/wt and PyMT/huMPO-G Mice



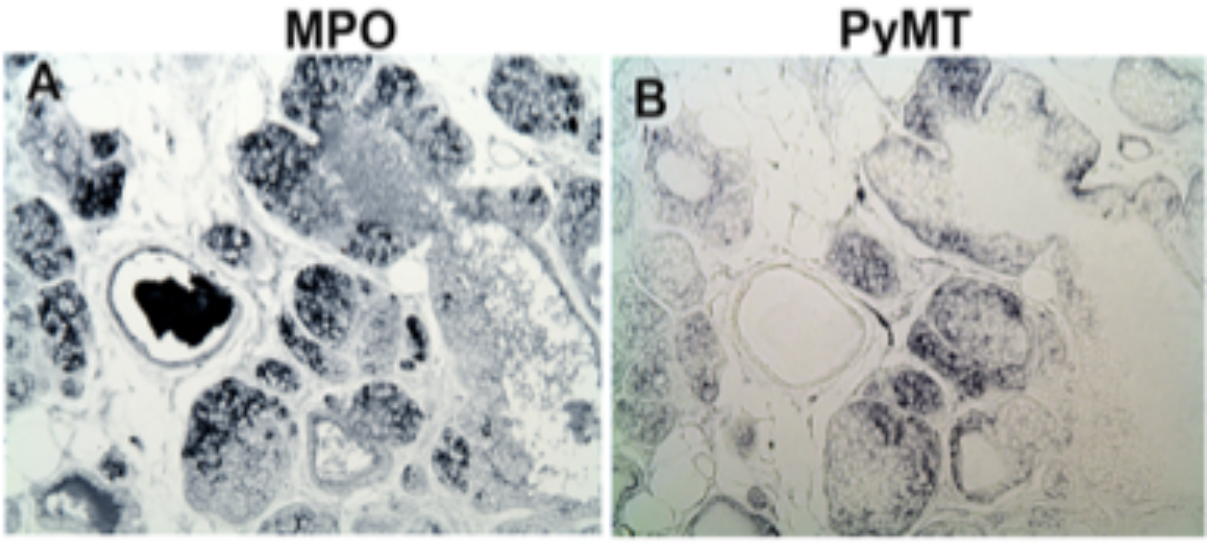
Human MPO (G-allele) results in a delay of mammary tumor growth in the PyMT model. Mice were examined once per week for the appearance of palpable tumors in either of the mammary glands. Once tumors were 2-3 mm in one dimension, tumor width and length were measured with calipers and tumor volumes were calculated. There was no difference in time of tumor onset or multifocal disease between PyMT/wt and PyMT/huMPO-G mice (see Table 1). Mean overall tumor burden and standard deviation is shown for PyMT/wt and PyMT/huMPO-G mice, 20 animals per group, t-test P= 0.06.

Figure 2 Human MPO Expression in Mammary Tumors from PyMT/hu-MPO-G Mice



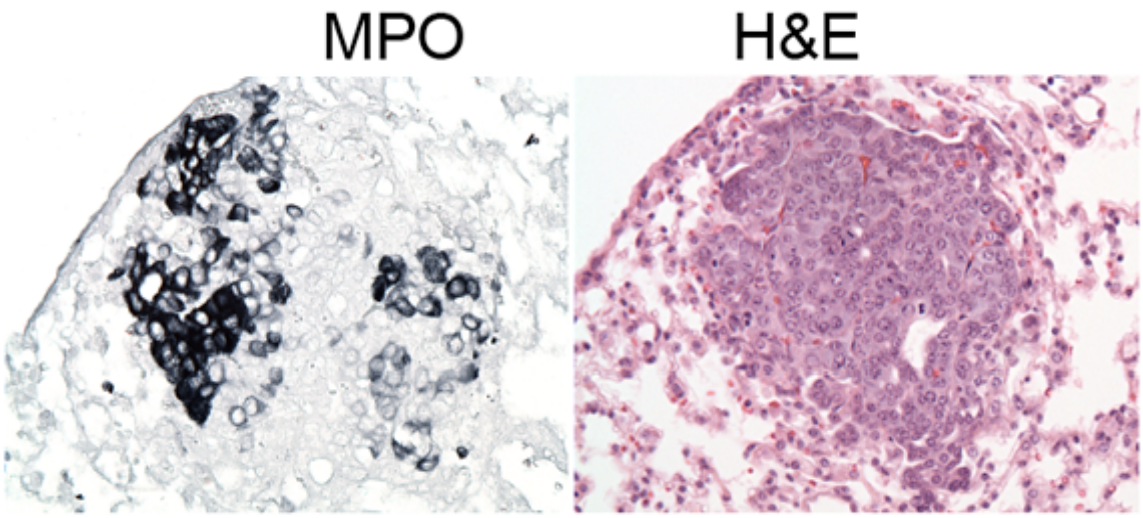
Human MPO immunostaining of early adenoma (week 15) PyMT/huMPO-G mammary tumors (D) and advanced adenocarcinoma (week 22) PyMT/huMPO-G tumors (A, B and C). Mammary tumor tissue immunostained with anti-human MPO antibody (Dako) (A, C and D) or adjacent section stained with H&E (B). (C) is enlarged from (A) showing MPO associated with the cytoplasm of tumors cells and expressed in a mosaic pattern.

Figure 3 Costaining for human MPO and PyMT in Mammary Tumors from PyMT/hu-MPO-G Mice



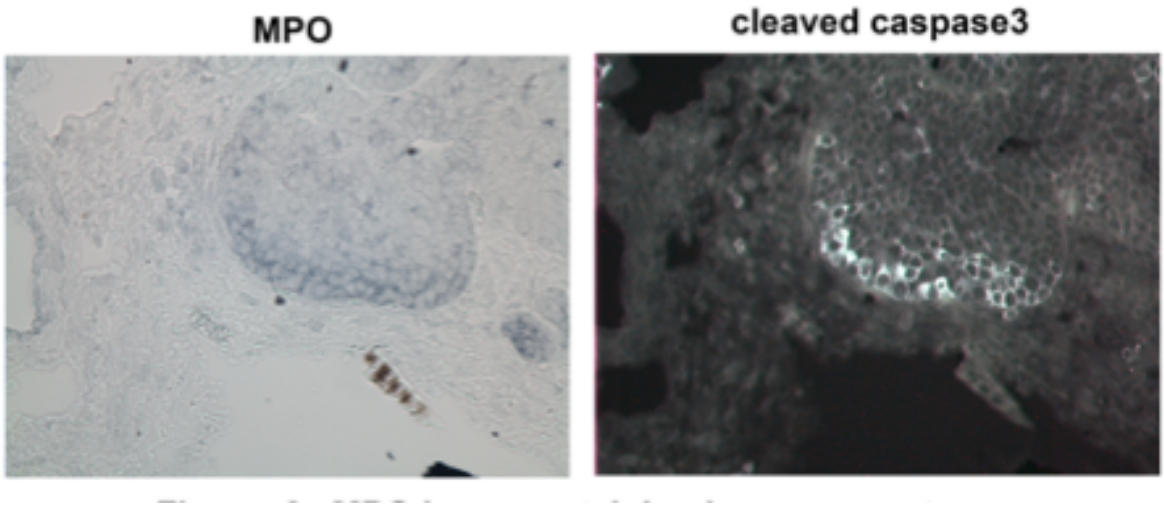
Adjacent sections from an advanced adenocarcinoma (week 23) were immunostained with anti-human MPO (Dako) or anti-PyMT (Novus).

Figure 4 Human MPO Expression in Lung Metastasis from PyMT/huMPO-G Mice



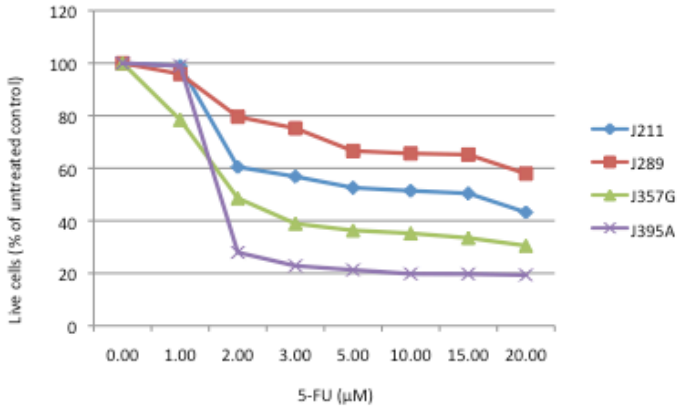
Lung tissue from a PyMT/huMPO-G mouse with advanced primary tumors and metastasis was stained with a polyclonal anti-human MPO antibody. Immunostaining shows mosaic MPO expression in some lung tumor foci. Shown is an example foran MPO-positive lung metastases.

Figure 5 MPO expression correlates with cleaved caspase 3



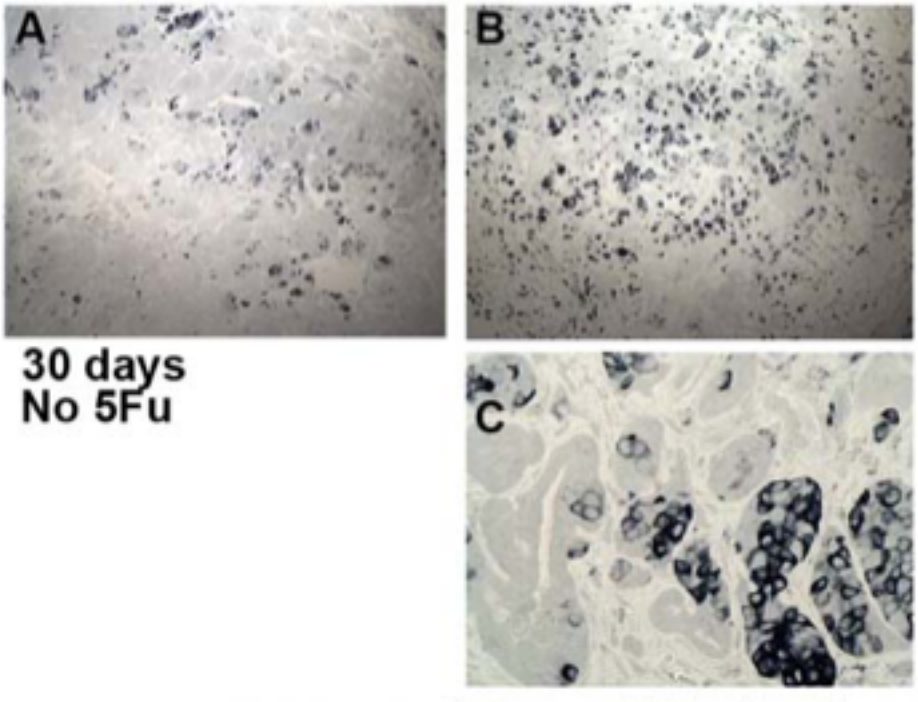
MPO immunostaining in a mammary tumor from a PYMT/huMPO-G mouse and doublestaining of the same section with antibody to cleaved caspase 3.

Figure 6 5-FU Cytotoxicity for Breast Cancer Cells



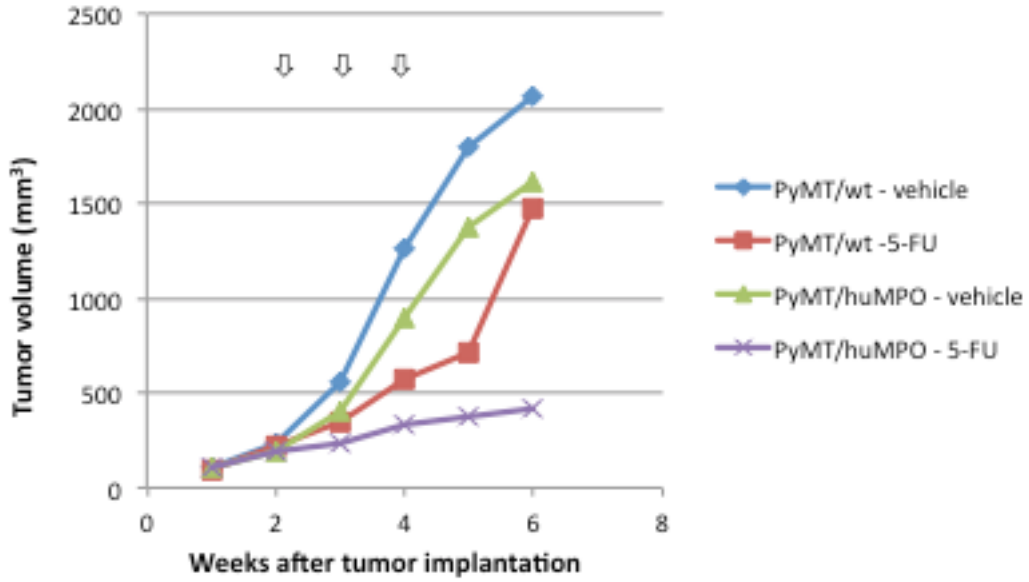
Cells were plated under cell culture conditions in 12 well plates and treated with 5-FU at the indicated concentrations for 72 hours. Cell viability was determined using the MTS reagent.

Figure 7 Effect of 5-FU on MPO expression in implanted PyMT/huMPO-G tumors



Small pieces of a PyMT/huMPO-G mammary tumor were subcutaneously implanted in the flank of a female C57Bl6 mouse. 25 days after implantation mice were treated with one dose of 5-FU (50mg/kg) or with vehicle. Mice were sacrificed 5 days later and tumor tissue was immunostained for human MPO. A, treated with vehicle; B, treated with 5-FU; C, enlarged from B.

Figure 8 Effect of huMPO expression on response to 5-FU chemotherapy



Female C57Bl6 mice were subcutaneously implanted with small pieces (5x5mm) of mammary tumors from PyMT/wt or PyMT /huMPO-G mice. Two weeks after implantation mice (8 animals per group) were treated with 5-FU (50mg/kg weekly sc for 3 weeks) or vehicle. In the grapg arrows indicate time of treatment. Tumor size was measured with calipers and tumor volumes were calculated as width^{2}xlength/2. The graph shows mean tumor volumes, SD were generally < 10% of the mean. Error bars are not shown for clarity. For PyMT/wt the difference between vehicle and 5-FU group was statistically significant at weeks 4 and 5 ($p<0.001$ by t-test) and week 6 ($p<0.05$). For PyMT/huMPO-G the difference between vehicle and 5-FU group was statistically significant at weeks 4, 5 and 6 ($p<0.001$ by t-test).